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SEROLOGICAL INVESTIGATIONS ON THE PHASEOLUS VIRUSES 1 AND 2

BY

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SEROLOGICAL INVESTIGATIONS ON THE PHASEOLUS VIRUSES 1 AND 2 [1])

by

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INTRODUCTION.

The common mosaic of French bean is a virus disease that has been known for a long time. SMITH (10) classified the causal agent as Phaseolus Virus 1. The fact that this virus may be transmitted by seed to the progeny of a diseased plant is undoubtedly one of the factors responsible for the world-wide occurrence of the virus (9).

Although some authors (7, 8) claim that Phaseolus Virus 1 may infect not only *Phaseolus vulgaris* but also other *Phaseolus* species and even *Vicia faba*, other investigators (3, 13) consider that *Phaseolus vulgaris* is the only host plant. In inoculation experiments the present authors failed to infect *Vicia faba* with this virus.

The symptoms caused by the virus depend on the bean variety and on environmental conditions. Some varieties react mainly with mosaic and light to heavy curling of the leaves; others show more or less severe necrosis (called "steengrauw" in Dutch) and some develop "black root" (12).

The yellow bean mosaic, classified by SMITH (10) as Phaseolus Virus 2, is less common. It has been described from the U.S.A. (5); in Europe it has been found, so far as we know, only in Belgium (11) and the Netherlands (12). This virus differs in two points from Phaseolus Virus 1; it is not transmitted by the seed of an infected plant, and it has a wider hostrange. Thus the virus infects several

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leguminous plants (5, 12), and in the U.S.A. it has been found that *Gladiolus* may carry it (6).

The symptoms of this virus also, are dependent on variety and on environmental conditions. Some bean varieties react with a more or less pronounced mosaic; others show top-necrosis, and sometimes symptoms are observed which are hardly distinguishable from the "steengrauw" caused by Phaseolus Virus 1.

Inactivation temperature, dilution end-point and longevity *in vitro* are the same for both viruses, *viz.*, about 58° C., 10^{-3} and between 24 and 48 hours respectively. Both viruses may be transmitted mechanically as well as by aphids. GROGAN and WALKER (4) have investigated in premunity studies the relationship between both viruses. Although the number of test plants was relatively low, GROGAN and WALKER concluded that bean plants infected with Phaseolus Virus 1 acquired premunity against Phaseolus Virus 2. Conversely, the last-mentioned virus protected bean plants, though to a lesser degree, against infection with Phaseolus Virus 1. From this more or less mutual exclusion effect it may be concluded that the viruses are related.

To obtain an exact picture of the relationship between two viruses an investigation of the serological behaviour of each is necessary, besides the premunity studies. The present writers therefore examined whether it was possible to obtain antisera against the Phaseolus Viruses 1 and 2, and further whether these viruses would react with the heterologous antisera.

In connection with this serological investigation it was considered desirable to study whether or not Phaseolus Virus 1 is present in clear-centrifuged sap of diseased French bean plants. This was done by an inoculation experiment, using *Phaseolus vulgaris* var. Beka as a test plant.

MATERIALS AND METHODS.

Phaseolus Virus 1 was isolated from a field-grown French bean plant showing common bean mosaic. Sap inoculations on *Vicia faba*, which were without result, showed that this virus was not contaminated with Phaseolus Virus 2. The virus was multiplied in the French bean variety Beka. Phaseolus Virus 2 was represented by a strain isolated in 1949 from a French bean plant which showed topnecrosis. Unlike the strain described in a former paper (12), which

caused only a very faint mottling in *Vicia faba* and *Pisum sativum*, this one was characterized by a distinct mosaic in these plants. The virus was maintained in *Phaseolus vulgaris* var. Beka and *Vicia faba* var. Driemaal Wit.

To obtain antisera against both viruses, rabbits were intravenously injected with sap from diseased plants, *viz.*, *Phaseolus* beans, var. Beka, infected with Phaseolus Virus 1 and broad beans, var. Driemaal Wit, infected with Phaseolus Virus 2 respectively. The plants were crushed in a mortar, the sap expressed by hand and filtered through filter paper. Afterwards the sap was dialyzed in cellophane for three or four hours in running tap water to free it from toxic substances which might be present in it. The dialyzed sap was injected without further treatment. The rabbits received four injections per week, with three days interval at the week-ends. Three rabbits were injected with Phaseolus Virus 1; two with Phaseolus Virus 2. Every animal received a total of about 45 ml during 12 injections, without disadvantageous consequences. One week after the last injection 50 ml of blood was taken from an ear vein of each animal. This was repeated during the next two days. The serum was separated from the blood in the ordinary way and was kept at —24° C.

Saturation of the sera was done with uncentrifuged sap from healthy Beka or Driemaal Wit plants. It was found that saturation took place best by using sap previously diluted in equal volume with saline. The Phaseolus Virus 1 antiserum proved to be saturated after mixing one volume of serum with 15 volumes of diluted sap; and Phaseolus Virus 2 antiserum after mixing one volume of serum with 24 volumes of diluted sap. The mixtures were kept at room temperature for 5 hours and then centrifuged. The clear supernatant liquids were used in the serological experiments, which were carried out according to the micromethod of VAN SLOGTEREN and cow orkers (2).

This method is carried out by mixing one drop of serum with one drop of sap on a cover slip. The cover slip is then mounted on a small glass ring that has been stuck on a microscope slide in the same way as a moist chamber is prepared. The serological reaction takes place in the hanging drop. After incubation at 37° C. the result of the reaction may be examined microscopically. In our experiments examination took place after 20 or 30 minutes.

Precipitation as well as agglutination tests were investigated.

For the first tests, the sap of diseased and healthy plants was centrifuged for 20 minutes at 12.000 r.p.m. The reactions were carried out in series, in which dilutions of saturated antiserum were combined with dilutions of sap. Normal serum, previously treated with sap from healthy plants (haricot and broad beans) in the same way as the antisera, served as check. Within 5 hours the mixtures antiserum-healthy sap showed a strong coagulation of plastids and other solid sap components, whereas the mixtures normal serum-healthy sap showed no coagulation. Saline, which was used as a diluent, served as second check.

The infection experiments were carried out on Beka plants that had just unfolded their first leaves. Inoculations were performed on one day by the same person. Carborundum, 500 mesh, was used as an abrasive.

Results.

§ 1. Serological reactions with Phaseolus Virus 1 antiserum.

The results obtained with the sera of the three rabbits injected with Phaseolus Virus 1 did not differ very much. Several dilutions of the saturated antiserum were made, running from undiluted to the dilution 1 : 64. The same series was made from normal serum, treated with the sap of healthy plants. The dialyzed and centrifuged sap of Beka plants infected with common bean mosaic and of healthy Beka plants was combined in the undiluted form and in the dilutions 1 : 2 and 1 : 4, with this series. This experiment was carried out late in the summer of 1950. The serum sap mixtures were kept at 37° C. for 20 to 30 minutes and then examined microscopically. It was found that all dilutions of the sap of healthy plants with the antiserum and normal serum series had remained clear; white, although rather faint, precipitates had formed in the mixture of the undiluted centrifuged sap of mosaic diseased bean plants with the antiserum range. A very faint reaction was obtained with one of the three saturated antisera in a dilution of 1 : 32, corresponding with a serological titre of $1/16 \times 1/32 = 1 : 512$. Thus calculated, each of the other two sera had a titre of 1 : 256.

It is remarkable that the diluted sap of the diseased plants showed no visible reaction. Normal serum mixed with the sap of diseased plants did not give a positive reaction.

In an experiment using undialyzed sap, it was found that spontaneous precipitates occurred in the mixtures serum-"healthy" sap. Conditions for obtaining good results in the precipitin reaction are thus to dialyze the sap previously and secondly to avoid dilution of the sap.

Furthermore, it appeared that saturation of the antiserum can best be done at room temperature. Keeping the mixture antiserum-sap of healthy plants at 37° C. for 3 hours may result in the destruction of the virus antibodies. Antiserum treated in this way did not show any positive reaction with the sap of diseased plants. Finally, it was found that the saturated antiserum stored at 1° C. may be kept only for two to three days. Serological activity proved to be lost after this period.

The results obtained lead to the conclusion that the quantity of virus in clear-centrifuged sap of mosaic diseased beans is rather small, as even the 1 : 2 diluted sap does not show a positive reaction. On the other hand the titre of the sera is rather high, or in other words the sera contain a considerable quantity of antibodies against the virus. Obviously much of the virus is sedimented during centrifugation. This conclusion was tested and confirmed in an inoculation experiment to be described in § 4.

The same antisera were also tested in the agglutination reaction. The dialyzed sap of healthy and diseased Beka plants was mixed undiluted and in the dilutions 1 : 2 and 1 : 4 with the saturated antiserum, which was diluted with different quantities of saline. Normal serum, previously treated with sap of healthy plants, and saline, served as checks. It was found that the sap of the diseased bean plants contained much starch, even when the plants were picked early in the morning. It is possible that the normal transport of sugars may be disturbed as a consequence of the disease. The starch grains showed a high tendency to agglutinate, which made the result of the serological reaction hard to judge. A centrifugation of the sap at low speed for one or two minutes before mixing with antiserum removed the bulk of the starch, without precipitating the other solid sap components. Agglutination of plastids was sometimes difficult to observe. It is noteworthy that it occurred distinctly in the sap of diseased plants grown in the glasshouse during August and September 1950 and April 1951, but that no positive agglutination tests were obtained in the sap of diseased beans grown during the winter months.

§ 2. Serological reactions with Phaseolus Virus 2 antiserum.

For the precipitin test, dialyzed and centrifuged sap, in this case of diseased broad bean plants, was used. The experiment was carried out in the autumn of 1950. The reactions with the undiluted sap of diseased *Vicia* plants were positive, even at high dilutions of the antiserum (both sera had a dilution end point of 1 : 32, which corresponds with a titre of $1/25 \times 1/32 = 1 : 800$. Furthermore, only the sap of diseased plants in the dilution 1 : 1 with saline reacted positively with the undiluted saturated antiserum.

The agglutination reaction of Phaseolus Virus 2 antiserum with sap of *Vicia faba* infected with this virus was very distinct (see Fig. 1). No disturbing starch was present in the sap. The saturated antiserum, diluted 1 : 16 with saline, still gave a positive agglutination reaction with undiluted sap of diseased broad bean. Thus the titre of this antiserum, defined in the agglutinin test, lays between 1 : 400 and 1 : 800. Normal serum and saline did not produce any agglutination of the plastids.

Contrary to the experience obtained in the study of Phaseolus Virus 1 anti-sera, all diseased *Vicia* plants tested showed a distinct positive reaction in autumn and winter as well as in spring.

The agglutination reaction of the saturated Phaseolus Virus 2 antiserum was further tested with the sap of Beka plants infected with this virus. This sap was freed from superfluous starch grains by a short low-speed centrifugation. It is noteworthy that the result of this reaction was also found to depend on the season in which the French beans were grown. In the autumn of 1950 and in the spring of 1951 positive reactions were obtained with the sap of Beka beans infected with Phaseolus Virus 2, whereas all tests with the sap of winter-grown infected bean plants remained negative.

§ 3. Study of the two viruses with the heterologous antisera.

Owing to the fact that the sap of Beka plants infected with one of the two viruses does not react visually with its homologous antiserum during winter time, one might suppose that in the case of a possible serological relationship between the Phaseolus Viruses 1 and 2 there might be a chance that the saturated Phaseolus Virus 1 antiserum would not give a reaction with the sap of Beka plants infected with Virus 2, and also that the saturated antiserum against

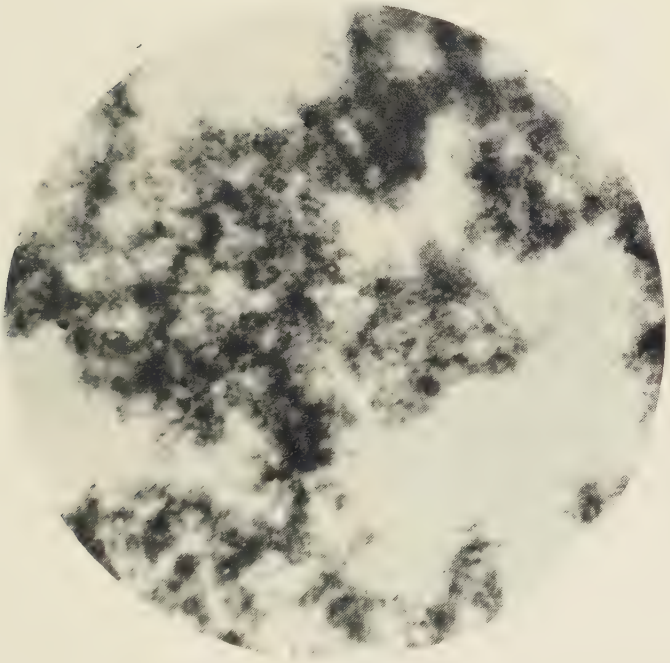

Fig. 1*a*. Sap of *Vicia faba* var. Driemaal Wit, infected wit Phaseolus virus 2, mixed with Phaseolus Virus 2 antiserum, saturated with sap of healthy *Vicia faba*.

Fig. 1*b*. Sap of healthy *Vicia faba* var. Driemaal Wit, mixed with Phaseolus Virus 2 antiserum, saturated with sap of healthy *Vicia faba*.

Virus 2 would not react with the sap of Beka plants infected with Virus 1. This proved to be true during the winter months. However, faint positive agglutination reactions were obtained during April 1951. The antiserum against Phaseolus Virus 1 always caused a distinct positive agglutination of plastids in the sap of *Vicia faba* infected with Phaseolus Virus 2. This shows that there exists a serological relationship between the two viruses.

§ 4. Inoculation experiment.

In order to verify the indication that very little virus is present in the centrifuged sap of Beka plants infected with Phaseolus Virus 1 (§ 1), an inoculation experiment was carried out during the autumn of 1950. The infectivity was estimated of: (1) sap of diseased Beka plants; (2) sap of the same plants clear-centrifuged for 20 minutes at a speed of 12.000 r.p.m.; (3) the sediment, obtained after centrifugation, resuspended in distilled water to the original volume of sap. As bean leaves do not react with local lesions after inoculation with Phaseolus Virus 1, the virus concentrations in these three objects were estimated in dilutions, *viz.*, undiluted, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. The undiluted sap and the dilutions were each inoculated into the primary leaves of about 45 bean plants. The results of the experiment are summarized in Table I.

Table I. [1])

	dilutions				
	undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Untreated sap	32/45	17/48	12/45	2/45	0/44
Clear-centrifuged sap	0/43	0/46	0/41	0/45	0/39
Sediment, resuspended in water	41/44	25/44	6/45	0/44	0/43

1) The denominator shows the number of inoculated French bean plants; the numerator the number of plants showing symptoms.

It was concluded from this experiment that the clear-centrifuged sap was no longer infectious, the infectivity of the uncentrifuged sap having gone into the sediment. This result agrees with the results of the serological precipitin reactions, described in § 1.

Discussion.

It has been shown that it is possible to obtain antisera against the Phaseolus Viruses 1 and 2 by injecting rabbits intravenously with dialyzed sap of virus diseased plants. Phaseolus Virus 2 can be demonstrated easily in the sap of *Vicia faba* var. Driemaal Wit, infected with this virus, using the agglutination test. It is more difficult to prove that antibodies against Phaseolus Virus 1 are present in its antiserum, as the reaction is sometimes inhibited by a factor in the sap of *Phaseolus vulgaris* var. Beka. With respect to this it has been shown by Bawden and Pirie (1), that by milling the fibre of tomato plants in a triple roller mill a substance may be liberated that inhibits precipitation of the tomato bushy stunt virus with its antiserum. Furthermore, Bawden and Pirie (1) showed that an analogous inhibitor is present in *Phaseolus vulgaris* var. Canadian Wonder. In our case a factor is concerned which inhibits agglutination. This inhibiting effect is most strongly developed when the sap is used from plants which have been grown during the winter months. It remains uncertain in which constituent of the sap this inhibiting factor is localized.

The results of the agglutination tests between Phaseolus Viruses 1 and 2 and their heterologous antisera indicate that the two viruses are serologically related. The reactions gave no information about the degree of relationship, as the study of this was hampered by the presence in the sap of Beka plants of the above-mentioned factor which inhibits the agglutination reaction. It may also be concluded from the work of Grogan and Walker (4), who found the two viruses to have a certain mutual premunizing effect in French beans, that these viruses are related.

Furthermore, it was found in the autumn of 1950, with the help of precipitin tests, that very small quantities of virus are present in the clear-centrifuged sap of *Phaseolus vulgaris* var. Beka, infected with Phaseolus Virus 1. An inoculation experiment with clear-centrifuged Beka sap gave good evidence, that this is not due to the action of a precipitation inhibiting agent. Although the inoculations remained negative, it was found that the sediment, obtained after centrifugation of the sap, showed the same infectivity as the uncentrifuged sap.

Summary.

1. Antisera against the Phaseolus Viruses 1 and 2 were obtained by injecting rabbits intravenously with uncentrifuged, dialyzed sap from *Phaseolus vulgaris* var. Beka, infected with Phaseolus Virus 1 and from *Vicia faba* var. Driemaal Wit, infected with Phaseolus Virus 2.

2. These antisera gave faint precipitin reactions with the clear-centrifuged sap of plants infected with the respective viruses.

3. The antiserum against Phaseolus Virus 1 gave faint agglutinations with the sap of *Phaseolus vulgaris* var. Beka, infected with Phaseolus Virus 1 during autumn and spring. During the winter months all reactions remained negative.

4. Distinct agglutinations in the sap of *Vicia faba* var. Driemaal Wit, infected with Phaseolus Virus 2 were obtained, not only in autumn and spring, but also in winter time, after mixing the sap with the antiserum against Phaseolus Virus 2. The sap of *Phaseolus vulgaris* var. Beka, infected with the same virus, behaved in the same way as sap infected with Phaseolus Virus 1. All reactions of the infected saps with normal serum remained negative.

5. Distinct agglutination of the solid components in the sap of the broad bean variety Driemaal Wit, infected with Phaseolus Virus 2, occurred after mixing with antiserum against Phaseolus Virus 1. The antiserum against Phaseolus Virus 2 gave positive reactions with the sap of French bean plants, var. Beka, infected with Phaseolus Virus 1, only when the plants were grown in autumn or spring. During the winter the reactions remained negative. From these results it is concluded that the Phaseolus Viruses 1 and 2 are serologically related.

6. The sap of *Phaseolus vulgaris* var. Beka, infected with one of the viruses mentioned, apparently contains a factor which inhibits agglutination of the solid sap components after mixing with the saturated homologous or heterologous antiserum. The effect of this factor is very marked in the sap of plants grown during the winter months. The effect is less during spring and autumn, since rather faint reactions may be given at those times of the year. A similar factor is not present to any marked degree in the sap of *Vicia faba* var. Driemaal Wit.

7. Phaseolus Virus 1 proved to be associated to a large extent with the solid sap components.

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